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Application of Molecular Techniques to Elucidate the Influence of Cellulosic Waste on the Bacterial Community Structure at a Simulated Low Level Waste Site

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ABSTRACT

Low-level radioactive waste sites, including those at various U.S. Department of Energy (DOE) sites, frequently contain cellulosic waste in the form of paper towels, cardboard boxes, or wood contaminated with heavy metals and radionuclides such as chromium and uranium. To understand how the soil microbial community is influenced by the presence of cellulosic waste products, multiple soil samples were obtained from a non-radioactive model low-level waste test pit at the Idaho National Laboratory. Samples were analyzed using 16S rRNA gene clone libraries and 16S rRNA gene microarray (PhyloChip) analyses. Both methods revealed changes in the bacterial community structure with depth. In all samples, the PhyloChip detected significantly more Operational Taxonomic Units (OTUs), and therefore relative diversity, than the clone libraries. Diversity indices suggest that diversity is lowest in the Fill (F) and Fill Waste (FW) layers and greater in the Wood Waste (WW) and Waste Clay (WC) layers. Principal coordinates analysis and lineage specific analysis determined that *Bacteroidetes* and *Actinobacteria* phyla account for most of the significant differences observed between the layers. The decreased diversity in the FW layer and increased members of families containing known cellulose degrading microorganisms suggests the FW layer is an enrichment environment for these organisms. These results suggest that the presence of the cellulosic material significantly influences the bacterial community structure in a stratified soil system.

INTRODUCTION

The processing of nuclear materials, operation of nuclear reactors, research and development activities at government sites, hospitals, universities, and radiochemical and radiopharmaceutical manufacturers have led to the generation of a substantial amount of low-

level mixed radioactive and heavy metal wastes that have been disposed in pits, trenches, and other waste sites (2). Co-disposed with metals and radionuclides were large quantities of cellulose containing materials such as wood, paper towels, cardboard, cheesecloth, and other materials (53). These wastes result from glove box operations, decontamination, housekeeping, maintenance, and construction activities, and can constitute up to 90% of the volume of typical low-level waste (LLW) (60). While there are over 20,000 commercial users of radioactive materials (2), the Department of Energy (DOE) complex houses the majority of disposed LLW waste at sites including Savannah River, Hanford, Idaho National Laboratory (INL) and Nevada test sites (3). Prior to 2000, the DOE disposed of approximately 2 million cubic meters of LLW and has projected the disposal of an additional 10.1 million cubic meters by 2070 (3). Within the Subsurface Disposal Area at the INL alone, approximately 330 metric tons of U-238 have been buried with cellulose containing material (26, 31). While these LLW materials are generally classified as such due to their low radioactivity and metal concentrations, their large quantity suggests there is potential environmental concern if mobilization of these contaminants was to occur.

The mobility of heavy metals and radionuclides in the subsurface may be greatly affected by the decomposition of this cellulosic waste by cellulolytic or fermentative microorganisms. A number of soil microorganisms can degrade one or more lignocellulosic components (i.e. cellulose and hemicellulose) to their respective subunits, which include cellobiose, C-5 and C-6 sugars (i.e. xylose, mannose, and glucose) (7, 38, 43). The breakdown of cellulose itself may release the associated metals and radionuclides, potentially increasing their mobility. Additionally, fermentative bacteria can then use these cellulose breakdown products as carbon and energy sources producing a variety of fermentation products including short chain organic

acids, alcohols and hydrogen (20). These fermentation products may significantly influence contaminant mobility, since organic acids can chelate metals and radionuclides potentially increasing their mobility (8, 21, 27, 44, 47). On the other hand, the work of numerous investigators has shown that these same compounds can serve as the carbon and energy source for metal and sulfate reducing bacteria that reduce and precipitate the metals and radionuclides found at these sites (1, 7, 19, 30, 39, 40, 45, 48, 52, 56, 59).

To better understand interactions between the bacterial community, cellulosic waste, and contaminants at LLW sites, the bacterial community must first be identified. Little is known about the bacterial community structure at LLW sites as previous studies have focused on culture dependent techniques, the construction of small clone libraries, and Denaturing Gradient Gel Electrophoresis (19, 20). Therefore, this study aims to perform a larger in-depth molecular analysis of the entire bacterial community at one of these sites. Soil cores from a surrogate waste pit at the INL were collected and samples from four depths within the pit were analyzed using 16S rRNA gene clone libraries and high-density 16S rRNA gene microarrays (PhyloChip). The overall goal of this study was to determine how the presence of buried cellulosic waste influences the bacterial community structure found at a LLW site.

MATERIALS & METHODS

Site Description. The Cold Test Pit South (CTPS) is located at the DOE INL Radioactive Waste Management Complex (RWMC) about 50 miles west of Idaho Falls, Idaho. The CTPS was constructed in 1988 and filled with simulated wastes that conform to the historical disposal practices at the RWMC between 1953 and 1970 (58). The pit was constructed to provide a clean environment to test the implementation of innovative waste characterization, retrieval

technology, performance and operational testing of remedial action scenarios. Cardboard was used as simulated waste containers to promote rapid deterioration and simulate up to 35 years of burial in shallow land filled pits. The bottom of the CTPS was lined with a crushed sediment clay liner (Figure 1). The waste layer, designated as the wood waste layer, contains stacked cardboard boxes, drums of combustibles (scrap wood, cloth, paper, plastic and HEPA filters), metals (aluminum and steel), concrete, asphalt, glass, and simulated inorganic sludges (silica and carbonate based pastes). Evidence from previous activities in the CTPS suggests that most of the simulated waste forms were concentrated at the base of the pit between 2.4 and 4.9 m below grade. The simulated waste layer was then covered with an overlying fill soil layer using local unsaturated soil. Compaction over time reduced the size of the simulated waste layer to approximately 0.2 m.

CTPS Sampling. A truck mounted Powerprobe 9600TM (AMS, Inc., American Falls, ID) direct push sampling rig was used to obtain intact core samples from the CTPS. Soil cores spanning the depth of the pit were collected in sterile 3.2 cm diameter Lexan™ core tubes (Figure S1 Supplemental Material). Samples were placed in a cooler on ice for shipment to the INL laboratory where the samples were processed.

Lexan tubes were cut at four designated depths representing various layers of the pit (Figure 1). The four soil layers that were sampled were the overlying Fill soil layer (F), the Fill soil/Wood Waste interface (FW), the Wood Waste soil layer (WW), and Wood Waste/Clay interface (WC). Approximately 2.5 cm of soil was removed aseptically using a sterile spatula, then a sterile 50 ml conical centrifuge tube was used to subcore for samples from which DNA was extracted. For samples that were obtained at interfaces (FW and WC), the soil sample obtained spanned each of the upper and lower layers equally. Samples were stored at -20 °C

prior to DNA extraction. Triplicate soil samples were collected from each of the four soil layers for individual DNA extraction and molecular analysis.

DNA Extraction and 16S rRNA Gene Amplification. DNA was extracted using the PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocol for the first set of soil samples from each layer (5 g soil per sample). DNA was extracted using the UltraClean Soil DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocol for the second and third soil sample (0.3 g per sample) from each soil layer. Since the WW layer soil was high in humic content, an additional clean-up step using a sephadex-based spin column was used according to instructions provided (*illustra* MicroSpin G-25 columns, GE Healthcare, UK) to remove compounds that would inhibit amplification.

PCR amplification of 16S rRNA genes was performed using 50 □L reactions containing a final concentration of 1x PCR buffer, 0.01 mg/mL bovine serum albumin, 0.5 Units JumpStart REDTaq DNA polymerase, (Sigma-Aldrich, St. Louis, MO), 0.4 □M 8F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), and 0.4 □M 1492R primer (5'-GGTTACCTTGTTACGACTT-3') (Integrated DNA Technologies, Coralville, IA). The reactions were heated to 94°C for 10 minutes, followed by 30 cycles of 94° C for 1 minute, 52° C for 1 minute, and 72° C for 1 minute, with a final extension at 72° C for 10 minutes (Applied Biosystems, GeneAmp PCR System 9700). The amplicons were checked for the correct size on the Agilent 2100 Bioanalyzer with the Agilent DNA 7500 Kit (Agilent Technologies, Waldbronn, Germany).

Cloning and Sequencing. Triplicate clone libraries were created for each soil layer using the three individual soil samples and DNA extracts obtained. 16S rRNA gene amplicons were

ligated into the pCR2.1 vector using the TOPO TA Cloning Kit and transformed into Top10 competent *Escherichia coli* cells, using the instructions provided (Invitrogen, Carlsbad, CA). Transformants were plated onto Sigma S-gal/LB agar and individual colonies containing vectors with inserts were chosen based on black/white selection and used to inoculate 1mL 2xLB with kanamycin in deep well plates. The plates were incubated between 16 and 18 hours at 37°C. The plasmid DNA was purified as per manufacturer's protocol (Montage Plasmid MiniprepHTS Kit, Millipore). The average concentration of the plasmid DNA was between 100 – 300 ng/µL as determined using a NanoDrop, ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The purified plasmid DNA from one clone library of each of the four soil layers was sent to Idaho State University Molecular Research Core Facility (ISU MRCF) for sequencing. The purified plasmid DNA from the other two clone libraries of each of the four soil layers was sequenced at INL. At both locations, Sanger cycle sequencing reactions with dye-terminators were prepared using between 100 and 200 ng template DNA, 1 µL BigDye v3.1 (Applied Biosystems, Carlsbad, CA), and one of three primers: M13F (5'-GTAAAACGACGGCCAG-3'), 515F (5'-GTGCCAGCMGCCGCGGTAA-3'), or M13R (5'-CAGGAAACAGCTATGAC-3') in a reaction volume of 10 □L (primer concentrations were 3.2 pmol/□L at ISU and 5 pmol/□L at INL). Reactions were denatured at 96°C for 1 minute, followed by 40 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. At the ISU MRCF, excess reagents and dye were removed using Millipore™-seq plates (Millipore, Billerica, MA) and DNA was analyzed on an Applied Biosystems 3130 Analyzer (Applied Biosystems, Carlsbad, CA). At INL, excess reagents and dye were removed using Performa DTRPlates (Edge Bio,

Gaithersburg, MD) and DNA was analyzed on a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA).

Sequence Analysis. Individual clones were sequenced using the forward, internal, and reverse primers, M13F, 515F, and M13R, respectively. Vector sequences were removed before assembly. Contiguous sequences were assembled using Phrap (16, 17) to make full-length 16S rRNA gene sequences. Clones were trimmed to remove poor quality regions using Phred (22) (Q<20), NAST-aligned (10), and checked for chimeras with Bellerophon (25) all through the use of tools provided by Greengenes (12) (www.greengenes.lbl.gov). Non-chimeric sequences were compared to public databases in Greengenes and classified using the G2_Chip taxonomy classification system. All nucleotide sequences from clone library analyses were deposited in GenBank under accession numbers GQ262819-GQ264537.

16S rRNA Gene Microarray Analysis. Amplification of the 16S rRNA gene from one of the DNA extractions obtained from each of the four soil layers was performed using 2 µg per reaction. Hybridization and subsequent analysis on a 16S rRNA gene-based microarray (PhyloChip) was carried out as previously described (11). Duplicate microarrays were analyzed for each soil layer sampled. A probe pair was scored as positive if (1) the fluorescence intensity of the perfect match probe was at least 1.3 times greater than the intensity of the mismatch probe and (2) the difference between the perfect match and mismatch intensities were 130 times greater than the square of the background intensity. An OTU was identified as present if at least 92% of the probe pairs for a specific OTU were scored as positive ($pf \geq 0.92$). An OTU was scored as positive for a soil layer if the OTU met these criteria for both replicate microarrays of each layer. ARB (42) version 08.07.08prv and the SILVA 04.10.08 reference database were used for the production of neighbor joining phylogenetic trees and MeV (49) for the production of heat maps.

Statistical Analyses. Statistical differences between duplicate PhyloChips and triplicate clone libraries for each layer were evaluated by Unifrac (41). Unweighted Principal Coordinates Analysis (PCoA) and lineage specific analysis were performed using Unifrac software for both the clone library and PhyloChip NAST-aligned sequences. Before PCoA analysis, clone libraries were analyzed using DOTUR (54) (www.plantpath.wisc.edu/fac/joh/dotur.html) in which a 97% cutoff was used to group sequences into OTUs. A single representative sequence from each OTU was included in analysis to eliminate phylogenetic weighting. Shannon's and Simpson's diversity indices as well as rarefaction curves (Figure S2 Supplemental Material) for both the clone library and PhyloChip data sets were also calculated using DOTUR.

Quantitative PCR. Family-specific primers for the *Acidimicrobiaceae*, *Flexibacteriaceae, Streptomycetaceae* and KSA Unclassified families were designed using the PROBE DESIGN and MATCH PROBE applications in ARB (42) version 08.07.08prv. Primers were designed and tested using an ARB neighbor joining phylogenetic tree with all sequences detected by both PhyloChip and clone library analyses. Each family-specific primer was paired with a general bacterial primer (Table S1 Supplemental Material). All primer pairs were determined to be highly specific to the target family (data not shown). Triplicate DNA extracts of each soil layer were diluted to the concentration used for amplification of clone library analysis. Equal volumes of each of the diluted DNA extracts were pooled for each soil layer. A two-step amplification using 5 nanograms of template DNA from each soil layer was carried out using the Rotor-Gene™ SYBR® Green PCR Kit (QIAGEN, Inc., Valencia, CA). An initial activation step of 95°C for 5 minutes, 35 cycles of a denaturation at 95°C for 5 seconds and a combined annealing/extension step at 60°C for 10 seconds was performed when using the *Acidimicrobiaceae*, *Flexibacteriaceae* and KSA Unclassified specific primers. Analysis with the

184 *Streptomycetaceae* specific primers had an increased combined annealing/extension temperature
185 of 65°C. Triplicate samples were analyzed for each soil layer using each set of family-specific
186 primers. Results are reported as 16S rRNA gene copy number per nanogram total DNA
187 extracted.
188
189 RESULTS
190 **Clone Library and PhyloChip Analyses.** A total of 448, 431, 382, and 458 clones were
191 obtained from the F, FW, WW, and WC layers, respectively, after sequences were trimmed,
192 aligned and screened for chimeras. The complete clone library of the simulated LLW site
193 contained 1719 clones. Analysis of sequences followed the "standard operating procedure for
194 phylogenetic inference" (46) regarding sequence alignment and phylogenetic tree building where
195 applicable. The triplicate clone library results for each layer were evaluated using Unifrac and
196 were determined not to be significantly different ($p \geq 0.2$). Therefore, the triplicate libraries for
197 each layer were combined and considered as one complete library for this study.
198 Duplicate PhyloChip analyses performed for each layer were also evaluated using
199 Unifrac, determined not to be significantly different ($p \geq 0.2$), combined, and also reported as
200 one data set for each layer. A total of 717, 1356, 1567, and 1582 unique OTUs were scored as
201 positive in the F, FW, WW and WC layers, respectively.
202 **Bacterial Community Structure.** Both the clone library and PhyloChip results indicated that
203 the bacterial community profile changed with depth when viewed at the phylum level. Clone
204 library analysis revealed that *Proteobacteria* were dominant in all four layers accounting for 29,
205 28, 35, and 56% of the F, FW, WW, and WC layer total clones, respectively (Figure 2A).
206 Twelve phyla were detected in the F layer by clone library analysis, with the *Proteobacteria*,

Actinobacteria and *Gemmatimonadetes* phyla comprising the majority of the total clones detected. These three phyla represented 332 of the 448 F layer clones or 74%. The FW layer contained clones from 10 different phyla, the least of any of the layers. The FW layer was comprised mostly of clones within the *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes*. The *Actinobacteria* and *Bacteroidetes* combined represented 60% of the total FW layer clones. This was a significant increase in *Bacteroidetes* clones from the F layer as they were 34% of the total FW layer clones and only 1% of the total F layer clones. The WW layer contained clones from 13 different phyla, the most of any layer, and the WC layers contained clones from 12 different phyla. Additionally, both layers were comprised mainly of *Proteobacteria*, *Bacteroidetes,* and *Acidobacteria*. These three phyla represented 286 clones, 74% of the total WW layer clones, and 379 clones, 83% of the total clones in the WC layer.

The PhyloChip data also indicated a change in community profile with depth and showed greater numbers of unique OTUs with increasing depth (Figure 2B). Though the number of unique OTUs changed with depth, four phyla were consistently dominant, and in similar ratios to each other, in all soil layers. The *Proteobacteria*, *Firmicutes*, *Actinobacteria,* and *Bacteroidetes* accounted for approximately 77, 84, 82, and 81% of the total OTUs detected by PhyloChip analysis in the F, FW, WW, and WC layers, respectively. In each layer, the *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* comprised approximately 50%, 15%, 11%, and 6%, respectively, of the total OTUs detected by the PhyloChip in each soil layer.

A comparison at the OTU level between methods indicates that the PhyloChip detected significantly more OTUs than the clone libraries in all soil layers. Clone library analyses detected 191, 173, 217, and 252 unique OTUs in the F, FW, WW, and WC layers, respectively compared to the PhyloChip analyses which as previously mentioned detected 717, 1356, 1567,

and 1582 unique OTUs in the same layers. A total of 2002 unique OTUs were detected by the entire study. Of these, only 10% were detected by both the clone library and PhyloChip (Table S2 Supplemental Material). Another 10% were detected by the clone library only while the remaining 80% were detected by the PhyloChip only.

Bacterial Community Diversity. Shannon's and Simpson's indices both indicated greater diversity in all four soil layers by PhyloChip analysis than by clone library analysis (Table 1). The Simpson's indices calculated for both methods demonstrated a similar trend in which overall the F and FW layers had the least diversity, while the WW and WC layers had the greatest diversity.

Shannon's indices calculated using the clone library data indicated there was no significant difference in diversity between soil layers. Conversely, Shannon's indices calculated with the PhyloChip data suggested there were significant differences in diversity between layers. Shannon's indices based on PhyloChip data determined that the FW layer had the least diversity, followed by the F layer, while the WW and WC had the greatest diversity.

Soil Layer Stratification. PCoA was performed with both the clone library and PhyloChip community data sets and the results suggest that there were significant differences between the bacterial communities with depth (Figure 3). The clone library data (Figure 3A) and PhyloChip data (Figure 3B) were first analyzed separately and yielded similar results. Triplicate clone libraries and duplicate PhyloChips for each soil layer clustered with themselves, again confirming the similarities between the replicates. When comparing soil layers, the WW and WC layers grouped closely together, while the F and FW layers clustered independently from the other layers. Not surprisingly, when the clone library and PhyloChip data sets were combined and analyzed, the method used to identify the community appeared to influence the clustering of

the data more heavily than the soil layer, since the PhyloChip data sets clustered together and independently of any of the clone library data (Figure 3C). The clone library data still demonstrated the same trend seen in Figure 3A: the F and FW layers each clustered by themselves, while the WW and WC layers clustered together.

Lineage specific analysis of the clone libraries was performed with Unifrac to determine which phyla were responsible for the differences between layers observed in the PCoA analysis. Multiple branch nodes were evaluated and it was determined that groups within the *Actinobacteria* and *Bacteroidetes* phyla were responsible for the majority of significant differences between layers (p value < 0.05). Unifrac could not support lineage specific analysis with the PhyloChip data, due to the large number of sequences. Because *Actinobacteria* and *Bacteroidetes* phyla are known to contain cellulose degrading microorganisms (43) and were identified as groups accounting for much of the change in bacterial community structure with depth, they were evaluated further to identify how they changed with depth. While the *Proteobacteria* also accounted for some of the changes identified by lineage specific analysis, the majority of these *Proteobacteria* clones were identified and categorized by Unifrac as only "suggestive" (p value 0.05-0.1) and thus less statistically significant.

***Actinobacteria* and *Bacteroidetes* Phyla.** There were 123, 113, 10 and 27 clones identified as belonging to the *Actinobacteria* in the F, FW, WW, and WC layers, respectively, corresponding to 28, 26, 3, and 6% of the total clones detected in each layer. Results indicate a difference in the *Actinobacteria* community structure with depth when viewed at the family level. In particular, four families showed significant changes with depth based on clone abundance: *Acidimicrobiaceae*, *Glycomycetaceae*, *Micromonosporaceae* and *Streptomycetaceae* (Figure 4,

Figure S3 Supplemental Material). Of these four families, two were chosen for additional quantitative analysis using 16S rRNA gene family-specific primers.

Lineage specific analysis identified *Acidimicrobiaceae* as responsible for some of the differences seen with the F layer when compared to the other three layers. The *Acidimicrobiaceae* family contributed 33% of the total *Actinobacteria* clones and 8.9% of the total clones detected in the F layer. An approximate 10-fold decrease in the percentage of *Acidimicrobiaceae* clones was observed between the F and FW layers (Figure 4A). No *Acidimicrobiaceae* clones were detected in the WW layer and only 3 were detected in the WC layer, accounting for less than 1% of the total clones detected. The PhyloChip, however, detected the presence of *Acidimicrobiaceae* OTUs in all four soil layers suggesting they are present throughout. The quantitative PCR data confirm the trends observed based on clone library analysis and also supports the Phylochip results as it detected the presence of *Acidimicrobiaceae* in the WW layer where no clones were identified. The *Streptomycetaceae* family had an approximately 40-fold increase in clone abundance between the F and FW layers (Figure 4B). This increase was followed by significant decreases between the FW and WW layer. The quantitative PCR analysis also identified a significant increase between the F and FW layers in which approximately a 100-fold increase was observed in *Streptomycetaceae* 16S rRNA gene copy number per nanogram total DNA. This was also followed by a significant decrease between the FW and WW layer. However, between the WW and WC layers a decrease in *Streptomycetaceae* 16S rRNA gene copy number per nanogram total DNA was observed while the clone libraries detected no clones in the WW layer and only one clone in the WC layer. The PhyloChip detected a large increase in the number of unique OTUs between the F layer and all other layers.

In the *Bacteroidetes* phylum, 5, 146, 93, and 69 clones were detected in the F, FW, WW, and WC layers, respectively, contributing approximately 1, 34, 24, and 15% of the total clones detected in these layers. This significant increase in the number of *Bacteroidetes* clones between the F layer and the other three layers partially explains how this phylum contributes to the observed stratification between layers. Four families in particular showed significant changes in clone abundance with depth and were identified by lineage specific analysis as contributing to the stratification between layers: *Crenotrichaceae*, *Flexibacteriaceae*, *Sphingobacteriaceae*, and KSA Unclassified clones (Figure 4, Figure S3 Supplemental Material). Of these four families, two were chosen for additional quantitative analysis using 16S rRNA gene family-specific primers.

No *Flexibacteriaceae* clones were detected in the F or FW layers, though the PhyloChip and quantitative PCR detected their presence in both layers. *Flexibacteriaceae* clones accounted for 5.5% and 5.0% of the WW and WC layer total clones, respectively (Figure 4C). Quantitative PCR analysis detected a decrease in *Flexibacteriaceae* 16S rRNA gene copy number per nanogram total DNA between the WW and WC layers, but a greater decrease than was observed by clone library analysis. The PhyloChip detected a greater number of unique OTUs within the WW and WC layers when compared to the other two layers.

KSA Unclassified clones detected in the F layer based on clone library analysis, accounted for only 0.9% of the total clones (Figure 4D). An approximate 7-fold increase in clone abundance was observed between the F and FW layers followed by a significant decrease in the WW and WC layers. Interestingly, the PhyloChip only detected one unique OTU that was present in all four soil layers. The quantitative data supports the trend observed by clone library analysis in which there was an increase in KSA Unclassified 16S rRNA gene copy number per

nanogram total DNA in the FW layer followed by a significant decrease in the WW and WC layers. It also detected this family in all four soil layers which supports the PhyloChip results.

Potential for Cellulose Degradation. To gain a better understanding of the potential role of the *Actinobacteria* phylum in response to the presence of cellulose, families were evaluated based on whether or not they had at least one significant change between two soil layers. A significant change was defined as at least a 4-fold increase or decrease in clone numbers, which coincides with approximately a 1% change in total clone abundance, between any two layers. Thirteen families out of 33 detected met this criterion: *Acidimicrobiaceae*, *Microthrixineae*, *Frankiaceae*, *Glycomycetaceae*, *Kineosporaceae*, *Microbacteriaceae*, *Micromonosporaceae*, *Streptomycetaceae*, *Thermomonosporaceae*, *Rubrobacteraceae*, and three unclassified families. These families were then differentiated based on their potential capabilities to degrade cellulose. Those that had been reported in the literature to be known cellulose degraders, cellobiose utilizers, or suggested to be cellulose degraders were grouped together as reported and implied cellulose degraders (4, 5, 9, 15, 34-37, 43, 50, 61). Those families that have never been shown to degrade cellulose, utilize cellobiose nor suggested to be able to do so were also grouped together as non-cellulose degraders. These groups were then compared in terms of their abundance and relative diversity with depth.

The clone abundance of the non-cellulose degrading group was highest in the F layer, accounting for 18.3% of the total clones detected in this layer, and decreased approximately 5-fold between the F and FW layer (Figure 5A). There were only 3 clones from this group in the WW layer and 7 clones in the WC layer accounting for less than 2% of the total clones in both layers. Conversely, the number of clones of the reported and implied cellulose degrading group was highest in the FW layer increasing 6-fold in abundance between the F and FW layer. This

group accounted for 17.9% of the total clones detected in the FW layer, decreasing in abundance in the deeper layers accounting for 1.6% of the total clones in the WW layer and 3.5% of the total clones in the WC layer. The greatest relative diversity, identified by clone library analysis, also correlated with the soil layer in which the greatest clone abundance was detected (Figure 5B). This was the F layer for the non-cellulose degrading group and the FW layer for the reported and implied cellulose degrading group. The PhyloChip also detected the greatest number of unique OTUs in the F layer for the non-cellulose degrading group, and in the FW layer for the reported and implied cellulose degrading group (Figure 5C). However, the change in the number of unique OTUs detected by PhyloChip analysis and relative abundance between all four layers was not as great as indicated by the clone libraries suggesting clone libraries may be more sensitive to significant changes in populations than the PhyloChip. Interestingly, the PhyloChip detected a greater number of unique OTUs within the reported and implied cellulose degrading group than the non-cellulose degrading group in all four layers. This may be due to an underestimate of the reported and implied cellulose degrading group's presence and diversity by the clone libraries, or may be due to a larger number of probes for this group found on the PhyloChip therefore increasing its chance of detection.

Unlike the *Actinobacteria*, all of the families that showed significant differences between layers contain known cellulose degraders (23, 24, 29, 32, 33, 43), except for the KSA Unclassified family of which no metabolic capabilities could be found in the literature. Regardless, the large number of reported and implied cellulose degrading *Bacteroidetes* families detected by clone abundance and PhyloChip analysis in the FW, WW, and WC layers suggests that there is potential for cellulose degradation in these layers.

DISCUSSION

Clone Library and PhyloChip Comparison. Both the clone library and PhyloChip analyses yielded valuable information about the bacterial community structure and diversity at the CTPS. While 1719 clones is a substantial clone library data set, the results of the PhyloChip analyses demonstrate that even with a large number of clones, the results barely depict the total diversity that was found at the CTPS as almost 80% of the total OTUs observed were detected by the PhyloChip only. The PhyloChip's sensitivity to low abundance OTUs is useful in identifying rare members of the community that may play a key role in the environment but are not present in high numbers. Still, the clone libraries detected 203 OTUs that the PhyloChip did not detect, and also provide insight into the potential abundance and dominance of these organisms at the CTPS making it a valuable method to use as well.

Similar to previous studies in which both PhyloChips and clone libraries were used, the PhyloChip detected greater overall diversity and number of unique OTUs (6, 11, 18, 51, 57). As previously mentioned, there were OTUs and even entire families detected through clone library analysis that were not detected by the PhyloChip. This may be due to poor hybridization with the probe, a sequence having a stronger affinity to the mismatch probe, or the absence of these sequences in the database when the probes were designed. It is also important to point out that when comparing the presence or absence of a specific OTU between the four soil layers, there was a low percentage of matches between the two methods. While it was not surprising that a unique OTU was detected only by the PhyloChip in a soil layer, it was surprising to observe the number of unique OTUs detected in some layers by the clone libraries only and in other layers by the PhyloChip only. This further supports the value of using these two methods to

complement each other to gain more information about the bacterial community and may be especially important in studies where one specific OTU or organism is focused on.

In addition to the molecular analyses discussed in this study, six bacterial isolates (members of the genera *Pseudomonas, Pedobacter, Streptomyces, Flavobacterium, Serratia, Cellulomonas*) were obtained from cellulose degrading enrichments inoculated with the soil from the FW, WW, and WC layers (Supplemental Information). The results of these cultivation studies can be compared to both the clone library and PhyloChip results to further demonstrate the differences between these two methods. All six isolates were detected at the family level by PhyloChip analysis in all three soil layers (Table S3 Supplemental Material). As the PhyloChip detects a great amount of diversity and large number of community members, it is not surprising that it would detect all six families in all soil layers. Meanwhile, clone library analyses detected some of these families, such as *Enterobacteriaceae* containing the *Serratia* sp. isolate and *Sphingobacteriaceae* containing the *Pedobacter* sp. isolate, in layers from which they were not isolated. This suggests that either these organisms were present and we were unable to culture them, or a different member of the family was present. While it is not surprising that there are soil layers in which these organisms are present but we were unable to culture them, it is interesting that a few of the isolates were cultivated from soil layers in which clone library analyses did not detect the presence of their families. For example, in the WW layer the clone libraries did not detect any members of the family *Streptomycetaceae*. However, a *Streptomyces* sp. was isolated from the WW soil layer and the PhyloChip confirms the family's presence. If only clone library analysis had been conducted, the results would suggest that there were no members of this family present in this layer. These results further demonstrate limits of clone library analysis and its potential to miss much of the diversity present at the site.

The results of this study show that the PhyloChip detects greater diversity which provides a more complete picture of the community structure and is important in identifying rare members of the community that may play an important functional role at the site. However, it is limited by the fact that in its current state it is not a quantitative method. Therefore, it cannot be used to determine which members of the community are more abundant and will not detect changes in abundance between soil layers. Also, the PhyloChip does not appear to be as sensitive to small changes within the community as seen with the *Actinobacteria* and *Bacteroidetes* phyla.

The clone libraries are semi-quantitative and begin to address which members of the community are abundant. Quantitative analysis performed for select families within the *Actinobacteria* and *Bacteroidetes* phyla support the data obtained by clone library analysis suggesting that such a large clone library dataset provides better confidence in the quantitative aspect of the clone library results. Still, as some differences were seen in the results of the quantitative PCR and clone library analyses, there are biases in the construction and analysis of clone libraries that limit its ability to be truly quantitative. On the other hand, they are more sensitive to changes within the community structure than the PhyloChip which is an additional advantage to using clone library analyses.

Low-Level Waste Site Microbial Communities. A total of 2002 unique OTUs were detected by both methods combined in all four soil layers and the dominant phyla observed (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria, Firmicutes*) were similar to those in other soil studies (14, 28, 55). Additionally, at least one of the methods used in this study detected the class, and in most cases, the family containing multiple genera identified in previous studies (including *Bacillus, Pseudomonas, Citrobacter, Clostridia, Azospira, Quadricoccus, Brevundimonas* and *Trichococcus*) focusing on LLW sites where culture techniques and small

clone libraries were used to characterize the bacterial community thus confirming their results (19, 20). The molecular techniques used in this study identified significantly more members of the bacterial community than previous studies. For example, Fox et al. 2006 (19) identified 8 distinct RFLP sequences from 29 clones in their low-level waste microbial community batch studies and even in the enrichments established in parallel to this study only six isolates were cultured while 2002 unique OTUs were identified. While it is known that culture-based techniques only focus on a small fraction of the microbial community, the findings of this study put into perspective how small a fraction that may be.

Influence of Cellulose on the Bacterial Community. Significant changes in the community structure and dominant phyla were observed with depth at the CTPS by both clone library and PhyloChip analyses suggesting the presence of cellulosic waste significantly influences the bacterial community at this site. PCoA analysis also supports this hypothesis as it showed a stratification of the bacterial community occurring within the CTPS between the F, FW, and WW layers. The similarities observed between the WW and WC layer bacterial communities suggest that this part of the CTPS is not as stratified as in the shallower depths. This may be due to the presence of the clay lining in the bottom that allows for the retention of water at this depth decreasing stratification between the two soil layers.

The F layer had a low diversity overall, suggesting a more oligotrophic soil environment, most likely containing few carbon and energy sources likely supplied through downward transport during precipitation and snowmelt events. Additionally, the decrease in the number of phyla detected and low calculated diversity at the FW layer, suggests there may be a selective influence on the community at this depth where those bacteria with a certain metabolic advantage are dominant. The abundance of the *Actinobacteria* and *Bacteroidete*s in this layer as

458 well as specific families within these phyla that contain known or potential cellulose degraders,
459 suggests that cellulose may be the selective influence at this depth and cellulose degrading
460 microorganisms may have a metabolic advantage.

461 The WW layer of the CTPS contains large quantities of cellulosic materials. Therefore, it
462 was hypothesized that this layer would most likely enrich for cellulose degraders. In this layer,
463 both the clone library and PhyloChip results indicate the presence of families containing known
464 cellulose degraders, suggesting cellulose degradation may be occurring at this depth. However,
465 increased diversity was also observed in this layer suggesting that cellulose is likely broken
466 down and utilized by either cellulose degrading organisms themselves or by other bacteria that
467 rely on these breakdown products for growth. These products, readily utilized by a wide variety
468 of microorganisms, would support a greater diversity of microorganisms in this layer. When
469 compared to the WW and WC layers, the decreased diversity observed in the FW layer may be
470 due to selective pressures on microorganisms in this layer, such as a lack of trace nutrients that
471 may have been buried with the simulated waste, lack of retained water or retained breakdown
472 products, which lead to the observed decrease in diversity in the FW layer. It is also important to
473 note that while fungi were not studied here, we recognize that they may be catalyzing cellulose
474 degradation at this site, and therefore may be influencing the activity and diversity of the
475 bacterial community between the different soil layers.

476 While the presence of these microorganisms cannot be linked to metabolic function
477 directly and there may be other environmental variables besides cellulose influencing the
478 bacterial community structure, the results demonstrate the possibility of cellulose playing a role
479 in the changes in community structure with depth.

480 We hypothesized that the *Firmicutes* would be dominant at this site since this phylum
481 contains many known cellulose degraders (13, 43), are often dominant in soil environments (28),
482 and are spore-formers, which is likely advantageous when fluxes of water and nutrients into the
483 system are minimal. The PhyloChip detected a large number of *Firmicutes* OTUs in all four
484 layers demonstrating a large relative diversity of this phylum present; however, the clone
485 libraries detected only 24 *Firmicutes* clones total in all four soil layers and overall the number
486 decreased with depth. It is possible that members of this phylum are either not very abundant at
487 this site, or the extraction and cloning method was not optimal for these organisms.

488 While all four layers were dominated by *Proteobacteria,* this was not surprising since the
489 *Proteobacteria* is a large, well studied phylum containing many known members. Some
490 members of the *Proteobacteria* such as *Pseudomonas spp.* can carry out aerobic cellulose
491 degradation (43) and while they may play a role in cellulose degradation at this site as they were
492 detected by both methods, they did not change significantly with depth. Members of this
493 phylum, as well as other phyla that did not change significantly with depth, may play important
494 roles in other processes occurring in the soil such as metal cycling or the cycling of other
495 nutrients. This may have significance in future studies which will focus on the interactions
496 between the bacterial community and heavy metals and radionuclides found at this site.

497 **Significance and Future Studies.** The results of this study provide insight on how the presence
498 of cellulosic waste influences the bacterial community. This is the most in-depth study to date of
499 the bacterial community found at a LLW site. To the authors' knowledge, this is also the most
500 in-depth study to date using both clone libraries and PhyloChip analyses to identify the bacterial
501 community found in any one soil environment due to the large clone library size, numerous
502 PhyloChips analyzed and evaluation of the site at multiple depths. Multi-depth sampling, such

as that performed in this study, can identify potentially important changes in the microbial community that may otherwise be overlooked. This will lead to the ability to better define and identify the potential roles different microorganisms have in metal mobility at these LLW sites and better design remediation processes that may be needed at these sites in the future.

Specifically, the results presented here will provide an extensive baseline for future studies investigating how bacterial community structure and function changes as a function of cellulose utilization. Column studies are being used to potentially identify which groups of organisms may be playing a key role in heavy metal and radionuclide mobility in simulated LLW environments. In these studies the bacterial community at both the DNA and RNA level will be evaluated and geochemical parameters will be monitored. These analyses will aid in linking the bacterial community structure with the community function. The results presented here are the first step in better understanding the interactions between the bacterial community, cellulosic waste, and contaminants at LLW sites.

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709 Table 1. Shannon and Simpson's Indices Calculated Using Clone Library and PhyloChip Data
710 for Each Soil Layer

	Shannon's Index		Simpson's Index	
Layer	**CL** (95% CI)	**PC** (95% CI)	**CL**	**PC**
F	5.56 (±0.090)	6.21 (±0.062)	3.40E-03	6.00E-05
FW	5.61 (±0.093)	5.86 (±0.074)	3.10E-03	6.20E-05
WW	5.67 (±0.071)	7.10 (±0.040)	1.70E-03	3.70E-05
WC	5.72 (±0.084)	7.03 (±0.041)	2.20E-03	3.40E-05

711 CL, Clone Library; PC, PhyloChip; CI, Confidence Interval; F, Fill; FW, Fill Waste interface;
712 WW, Wood Waste; WC, Waste Clay interface.
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727 **Figure 1.** Schematic of the non-radioactive CTPS near the LLW site at the Idaho National
728 Laboratory where soil samples were obtained. Brackets indicate sampling points. F, Fill; FW,
729 Fill Waste interface; WW, Wood Waste; WC, Waste Clay interface.

730

731 **Figure 2.** The bacterial community viewed at the phylum level with depth at the CTPS. (A)
732 Percent abundance of each phylum as determined by clone library analysis with the total number
733 of clones for that layer listed at the top of each bar. (B) Number of unique OTUs identified
734 within each phylum based on clone library (CL) and PhyloChip (PC) analyses. F, Fill; FW, Fill
735 Waste interface; WW, Wood Waste; WC, Waste Clay interface.

736

737 **Figure 3.** Principal Coordinates Analysis (PCoA) of the (A) combined clone libraries, (B)
738 combined PhyloChip data, and (C) combined clone library and PhyloChip data. A 97% identity
739 cutoff was used to remove replicate sequences from the clone libraries before analysis. F, Fill;
740 FW, Fill Waste interface; WW, Wood Waste; WC, Waste Clay interface.

741

742 **Figure 4.** (A) *Acidimicrobiaceae* and (B) *Streptomycetaceae* families within the *Actinobacteria*
743 phylum and (C) *Flexibacteraceae* and (D) KSA Unclassified families within the *Bacteroidetes*
744 phylum that had significant changes with depth as viewed by PhyloChip and clone library
745 analyses. PhyloChip results are presented as a presence (black) absence (gray) heatmap for each
746 OTU detected within the family. Each row, marked (•), represents a unique OTU. An OTU was
747 determined present in a soil layer if the pf value was above or equal to 0.92 for both PhyloChips.
748 Clone abundance of each family is reported as the percent of the total clones detected per soil
749 layer. Quantitative PCR was performed using family-specific primers for amplification of the
750 16S rRNA gene. F, Fill; FW, Fill Waste interface; WW, Wood Waste; WC, Waste Clay
751 interface.

752

753 **Figure 5**. Focus group comparisons of *Actinobacteria* phylum. Families with a significant
754 decrease in clone number between at least two layers (ex. significant change between F and FW
755 layer) were categorized as either reported and implied cellulose degraders (families that are
756 previously known to be cellulose degraders, cellobiose utilizers, or have been suggested to be
757 potential cellulose degraders) or non-cellulose degraders (families that have not been shown in
758 the literature to degrade cellulose, cellobiose nor has it been suggested that they can). These two
759 groups were then compared based on (A) clone abundance and the number of OTUs detected by
760 (B) Clone Library and (C) PhyloChip analyses. F, Fill; FW, Fill Waste interface; WW, Wood
761 Waste; WC, Waste Clay interface.